The Repo homeodomain transcription factor suppresses hematopoiesis in *Drosophila* and preserves the glial fate

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Running title: The Repo transcription factor suppresses hematopoiesis

Keywords: *Drosophila*/Repo/Gcm/Glial cells/ hemocytes

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Abstract

Despite their different origins, *Drosophila* glia and hemocytes are related cell populations that provide an immune function. *Drosophila* hemocytes patrol the body cavity and act as macrophages outside the nervous system whereas glia originate from the neuroepithelium and provide the scavenger population of the nervous system. *Drosophila* glia are hence the functional orthologs of vertebrate microglia, cells of immune origin that move into the brain during development and become the resident macrophages of the nervous system. Interestingly, glia and hemocytes require the same transcription factor Glide/Gcm for their development. This raises the issue of how do glia specifically differentiate in the nervous system and hemocytes in the procephalic mesoderm. The Repo homeodomain transcription factor and pan-glial direct target of Glide/Gcm ensures glial terminal differentiation. Here we show that Repo also takes center stage in the process that discriminates between glia and hemocytes. First, Repo expression is repressed in the hemocyte anlagen by mesoderm-specific factors. Second, Repo ectopic activation in the procephalic mesoderm is sufficient to repress the expression of hemocyte-specific genes. Third, the lack of Repo triggers the expression of hemocyte markers in glia. Thus, a complex network of tissue-specific cues biases the potential of Glide/Gcm. These data allow us to revise the concept of fate determinants and help us understanding the bases of cell specification.

Significance statement

Distinct cell types often require the same pioneer transcription factor, raising the issue of how does one factor triggers different fates. In *Drosophila*, glia and hemocytes provide a scavenger activity within and outside the nervous system, respectively. While they both require the

Glide/Gcm transcription factor, glia originate from the ectoderm, hemocytes from the mesoderm. Here we show that tissue-specific factors inhibit the gliogenic potential of Glide/Gcm in the mesoderm by repressing the expression of the homeodomain protein Repo, a major glial-specific target of Glide/Gcm. Repo expression in turn inhibits the expression of hemocyte-specific genes in the nervous system. These cell-specific networks secure the establishment of the glial fate only in the nervous system and allow cell diversification.

Introduction

In the *Drosophila* embryo, lateral glial cells (called glia throughout the text, for the sake of simplicity) constitute the second major population of the nervous system and are necessary for neuronal development, function and survival. Typically, they insulate the central nervous system (CNS) upon forming the blood-brain barrier (BBB) and regulate neurotransmitter recycling, axon guidance or neural proliferation (Trébuchet and Giangrande, 2012). During development and upon injury, *Drosophila* glia also act as scavenger cells and help reshaping the nervous system. Thus, *Drosophila* glia behave like microglia, vertebrate immune cells of mesodermal origin that move from the yolk sac into the brain during development and provide the resident macrophages of the CNS (Logan and Freeman, 2007; Kurant, 2011). Outside the fly nervous system, hemocytes play a key role in cellular and humoral immunity. They can move very fast to patrol the organism and respond to a variety of challenges. The most represented subtype of hemocytes, called plasmatocytes, phagocyte microbes and sculpt tissues by clearing apoptotic cells during development (Meister and Lagueux, 2003).

In addition to sharing the immune function, glia and hemocytes express the same transcription factor, the atypical zinc finger protein Glial cells deficient/Glial cells missing (Glide/Gcm, Gcm throughout the text) (Mao et al., 2012; Cattenoz and Giangrande, 2013) at early stages of their development. Gcm is necessary and sufficient to induce gliogenesis and is required for hemocyte differentiation (see (Cattenoz and Giangrande, 2014) for a review). Thus, the same transcription factor works in functionally related cells that originate from the neurogenic ectoderm (glia) and from the procephalic mesoderm or PM (hemocytes). In the nervous system, Gcm induces the expression of the Reverse polarity (Repo) homeodomain containing transcription factor in all the glial cells. Repo is necessary for the execution of the glial differentiation program (Yuasa et al., 2003) and embryos lacking Repo do not express late markers (Halter et al., 1995), including the scavenger receptor Draper (Shklyar et al., 2014). As a consequence, *repo* mutant glial cells are not functional and have defective phagocytic activity (Shklyar et al., 2014).

The shared molecular pathway and role of glia and hemocytes call for a cell-specific mechanism triggering embryonic glia and blood differentiation in the correct tissue. We here show that mesodermal cues contribute to prevent glial differentiation in the hemocyte anlagen. The mesodermal transcription factor Twist induces the expression of *miR-1*, which in turn represses the expression of Repo. As a consequence, the gliogenic potential of Gcm is inhibited in the PM (Xiong et al., 1994; Halter et al., 1995; Yuasa et al., 2003), showing that the potential of a fate determinant relies on the cell-specific transcriptional landscape. The negative regulation of Repo in the hemocyte anlagen is crucial as Repo represses the hemocyte fate: when expressed in the hemocyte anlagen, it inhibits the expression of hemocyte-specific genes and the lack of Repo induces the expression of early hemocyte markers in the nervous system. Thus, Repo constitutes a major element in the pathway that discriminates between related but distinct scavenger fates.

Altogether, our work dissects the complex network that allows a single pioneer factor to affect different cell fates.

Results

The mesoderm-specific transcription factor Twist represses the expression of the Repo pan-glial protein

The Gcm transcription factor is expressed in the glial as well as in the hemocyte lineages, where it controls the expression of glial and hemocyte genes, respectively (Jones et al., 1995; Bernardoni et al., 1997; Bernardoni et al., 1998; Lebestky et al., 2000; Alfonso and Jones, 2002; Cattenoz et al., 2016). Since glia differentiate from the ectoderm and hemocytes from the PM, we hypothesized that tissue-specific factors regulate the expression of the Gcm targets in a cell-specific manner. Twist (Twi) is an early mesoderm-specific transcription factor and a potent mesodermal determinant (Baylies and Bate, 1996), we therefore asked whether it represses the expression of Repo, the most characterized glial-specific target of Gcm. Repo also represents the only transcription factor expressed exclusively in glia and in all glia (Halter et al., 1995).

To show that Twi inhibits Repo expression *in vivo*, we analyzed embryos in which we induced Twist expression ectopically (Gain Of Function or GOF), in the neural territory, as well as embryos that lack Twi expression (Loss Of Function or LOF) or express low levels of Twi.

First, the ectopic expression of Twi in the neurogenic region mediated by the *scabrousGal4* driver (*sca>twi*) (Mlodzik et al., 1990) significantly reduces the number of Repo positive cells in the ventral nerve cord from an average of 29.3 +/-1.1 cells per hemisegment in control to 8.6 +/-1.1 cells in *twi* GOF embryos (n hemisegments=10, n embryos=3, ANOVA $p=8.10^{-11}$) (**Table 1**,

Figure 1 A,B). Second, since the expression of Gcm in the mesoderm triggers gliogenesis at the expense of muscles (Bernardoni et al., 1998), we performed the same experiment in embryos that carry half a dose of Twi and found that this enhances the gliogenic potential of Gcm in the mesoderm. This data were obtained upon expressing Gcm with the *twistGal4* driver (*twi>gcm*) in *twi/+* heterozygous embryos (**Table 1**, **Figure 1C,D**). Third, although Twi is a major mesodermal determinant that induces severe and early defects when absent (Thisse et al., 1987), it is not absolutely required for the initial determination of the hemocyte fate (Spahn et al., 2014). This allowed us to analyze the few *twi* null embryos that reached relatively late stages and revealed the presence of the Repo protein in cells that express the early hemocyte marker Serpent (Srp) (no cell in control and an average of 8.9 +/-4.3 cells Srp and Repo positive per embryo *twi* LOF, n embryos=5, Wilcoxon (W) p=0.0038) (**Figure 1E,F'')**. Unless otherwise specified, low magnifications of all the figures show confocal projections whereas high magnifications of the insets shown single confocal sections, for the sake of simplicity. This explains why the labeling in the insets corresponds partially to that shown in the low magnification panels. Altogether, our results strongly suggest that the lack of Twi allows ectopic Repo expression in the hemocyte anlagen, the PM, hence biasing the gliogenic potential of Gcm in that territory.

We then asked whether over-expressing Gcm in its own domain of expression, the PM, leads to the differentiation of supernumerary hemocytes or whether it bypasses the molecular brake imposed by Twi, hence allowing ectopic Repo expression. For this purpose, we crossed a *gcmGal4* driver with a transgenic line expressing the Gal4 inhibitor Gal80 in glial cells, the other territory of Gcm expression (**Table 1**, *gcmGal4, repoGal80* or *gcm(hemo)Gal4)* (Lee and Luo, 1999), so as to confine Gcm overexpression to the PM (**Figure 1G).** *gcm(hemo)>gcm* embryos do display Repo expression in the hemocyte anlagen and this is a dosage dependent phenotype, the stronger the *UAS gcm* transgene, the higher the levels of Repo (**Figure 1H)**. Moreover, and in line with our

hypothesis, co-over-expressing Gcm and Twi (*gcm(hemo)>gcm + twi*) abolishes the induction of Repo expression in the PM (**Figure 1I**).

The fact that Gcm over-expression induces Repo expression in the PM could mean that glial differentiation simply requires higher Gcm levels than hematopoiesis. If that were the case, hypomorphic *gcm* mutant embryos should express hemocyte markers in the nervous system. The gcm^{34} mutation is an imprecise excision that still expresses the *LacZ* gene carried by the P element located at the *gcm* locus and results in low Gcm levels (Vincent et al., 1996). Neither gcm^{34} homozygous nor gcm^{34}/*Df(2L)132* transheterozygous animals (the *Df(2L)132* deficiency completely deletes the gene (Kammerer and Giangrande, 2001)) show Srp ectopic expression in the nervous system (**Figure 1J-L**). This excludes mere dosage dependency for the establishment of the glial vs. the blood cell fate and further supports the idea that tissue-specific factors are responsible for it.

In sum, the Twist mesodermal factor negatively affects the expression of the pan-glial transcription factor Repo.

The micro RNA *miR-1* inhibits Repo expression post-transcriptionally

The microRNA *miR-1* is a direct target of Twi expressed and required in the mesoderm (Biemar et al., 2005; Sokol and Ambros, 2005). We found that *miR-1* has two putative target sites in the *repo 3'UTR* (miRanda: http://www.microrna.org/microrna/home.do) (**Figure 2A**) and therefore explored the possibility that it acts post-transcriptionally on Repo. First, we found that animals lacking *miR-1* display ectopic Repo expression in the PM, similar to the *twi* embryos (**Figure 2B-D**). Second, we asked whether *miR-1* directly acts on the *repo 3'UTR* by co-transfecting S2 *Drosophila* cells with a *miR-1* expression vector and a luciferase reporter carrying either the *repo 3'UTR* or its own *3'UTR* (**Figure 2E**). By measuring the luciferase activity, we

found that *miR-1* specifically acts on the *repo 3'UTR* to repress *repo* expression **(Figure 2E-F**). Third, this negative control is abolished upon mutagenizing the two putative *miR-1* target sites (**Figure 2F**). Thus, *miR-1* inhibits Repo expression post-transcriptionally.

In sum, our data indicate that mesoderm-specific cues prevent Gcm from triggering Repo expression in the PM.

Repo is sufficient to repress the expression of hemocyte markers in the PM

The tight repression of Repo expression in the hemocyte anlagen suggests that gliogenesis is alternative to hemocyte differentiation. We therefore analyzed the effects of Repo ectopic expression in the PM upon using the *UAS-repo* transgene (Yuasa et al., 2003). *gcm(hemo)>repo* (or *repo* GOF) hemocytes are severely affected: many of them aggregate and show altered morphology as well as migratory defects (**Figure 3E,F)**. Moreover, they no longer express the late hemocyte marker NimC/P1, which is a scavenger receptor (Kurucz et al., 2007) (**Figure 3C,D**), and the expression of the early hemocyte marker Srp is severely downregulated (**Figure 3A'',A''',B'',B''').** The hemocytes express Srp at low levels. To quantify this phenotype, we measured the intensity of Srp labeling and found a significant difference between control and *repo* GOF hemocytes (control: 83.4 +/-3.9 arbitrary unit (AU, see materials and methods), *repo* GOF: 12.4 +/-1.5 AU; n=50 cells in 3 embryos, ANOVA $p=6.10^{-23}$). Of note, Srp is also expressed in the fat body and yet such expression remains unchanged in *repo* GOF animals (Hoshizaki et al., 1994) (**Figure 3A',B'**), showing that the hemocyte defects are specific and cell autonomous.

A more direct evidence for the specific effects of Repo on the *srp* gene was obtained by using a Gal4 plasmid that carries a fragment of the *srp* promoter specific to hemocytes and called *srp(hemo)>* (Bruckner et al., 2004). Co-transfecting S2 *Drosophila* cells with a Repo expression vector and the *srp(hemo)>GFP* plasmid severely reduces the expression of the GFP, and this is a

dosage dependent effect **(Figure 3I)**. Moreover, *srp(hemo)>repo* embryos display similar features than *gcm*(*hemo)>repo* embryos, with reduced number of hemocytes **(Figure 3J-K'')**. Indeed, we found 252.8 +/-27.4 hemocytes in control and 136.8 +/-19.8 in *repo* GOF embryo (n=7 embryos, ANOVA p=0.0028, counted on 30µm stacks of confocal images taken from stage 13 embryos (lateral views)). Of note, the presumptive hemocytes that ectopically express Repo with the *gcm(hemo)>* or with the *srp(hemo)* driver do not express late glial markers (as monitored by the Nazgul antibody (von Hilchen et al., 2010; Ryglewski et al., 2017) (**Figure 3E-F'',J-K'').**

The reduction in the number of hemocytes in *repo* GOF is due, at least partially, to enhanced cell death, as shown by the apoptosis marker cleaved death caspase-1 (DCP-1) (Song et al., 1997) (**Figure 3G-H''**, 9.1% +/-1.3 of hemocytes display co-labeling with DCP1 in control vs. 16.1% +/-2.1 in *repo* GOF embryos, n=7 embryos, ANOVA p=0.0150).

Altogether, the above data strongly suggest that the expression of the Repo pan-glial factor in the PM is detrimental to hemocyte differentiation and are also in line with the fact that Repo is not sufficient to induce the glial fate when ectopically expressed (Yuasa et al., 2003).

Given the ability of Gcm over-expression in the PM to induce Repo ectopic expression, we re-examined that phenotype, in order to understand the relative roles of the two transcription factors in blood and glial development. Interestingly, the over-expression of Gcm in the PM induces both Repo and Nazgul expression in the presumptive hemocytes (von Hilchen et al., 2010) (**Figure 4A-D'''**, 21.7%+/-3.4 of Repo positive hemocytes/embryo, n=4 embryos, W p=0.0105 and 53.4% +/-6.4 Nazgul positive hemocytes/embryo, n=3 embryos, W p=0.0318 in *gcm(hemo)>gcm*, compared to 0% in control). Moreover, the cells that express Repo also express the hemocyte marker Srp (**Figure 4E-E'''**) (Rehorn et al., 1996), at levels that are comparable to those found in wild-type embryos (the intensity of Srp labeling in hemocytes from control = 83.4 +/-3.9 AU and from *gcm(hemo)>gcm* = 73.1 +/-6.2 AU, n=50 hemocytes in 3 embryos, ANOVA p=0.22). Thus, Gcm

over-expression induces the expression of glial genes without blocking hemocyte differentiation. Since Srp constitutes an early hemocyte gene (Reuter, 1994; Bernardoni et al., 1997; Lebestky et al., 2000), we asked whether late hemocyte markers are also detected in those cells or whether hematopoiesis is blocked at its early stages. The hemocyte-specific scavenger receptor Croquemort (Crq) (Franc et al., 1996; Franc et al., 1999) co-localizes with the pan-glial maker Repo (**Figure 4F-F'''**), indicating a mixed glial and hemocyte phenotype. This finding is in accord with the expression/requirement of Gcm in both hemocytes and glia. Of note, we never observed Repo expression in *gcm(hemo)>gcm* hemocytes at larval stages, suggesting that the Repo expressing cells do not survive or that Repo expression is not maintained. Finally, because the *gcmGal4* driver is expressed transiently and early in the hemocyte lineages, we confirmed these data by using additional hemocyte-specific drivers: *srp(hemo)Gal4, hemolectinGal4* and *hemeseGal4* (Bruckner et al., 2004) (**data not shown**).

Repo represses the expression of hemocyte markers in glial cells

Given the ability of Repo to inhibit the hemocyte fate in the PM, we asked whether it also represses that fate in glial cells. In the simplest view, the lack of Repo could transform glial cells into hemocytes, as glia represent the resident macrophages of the nervous system. By analyzing the role of Repo first in ectopic glial cells and then in endogenous glia, we found that this transcription factor represses the expression of hemocyte markers.

First we found that Gcm expression throughout the neurogenic region (*sca>gcm)* triggers ectopic gliogenesis, whereas the same experiment in *repo* null embryos (*repo* loss-of-function, *repo* LOF) triggers ectopic Srp expression within the nervous system. (**Figure 5A-B'''**). To identify the cells expressing Srp ectopically, we needed a lineage marker that traces the glial cells in wild-type embryos and the presumptive glia in embryos lacking Repo. We hence analyzed

sca>gcm; repo LOF embryos that also carry the *repo-nuclearGFP* (*repo-nGFP*) transgene, which faithfully recapitulates the expression profile of Repo. Since Srp is a nuclear marker, using the nuclear GFP tagging we could show Srp/GFP co-localisation (**Figure 5A-B'''):** 3.5 +/-0.8 cells/hemisegment show Srp/GFP co-localisation in *sca>gcm;repo* LOF,*repo-nGFP* embryos as compared to 0 cells in *sca>gcm;repo-nGFP* embryos (n=6 hemisegments in 3 embryos, W $p=2.10^{-4}$). This indicates that the presumptive glia express Srp, as opposed to the possibilities that *repo-GFP* positive cells phagocyte Srp positive cells (Jones, 2005; Laneve et al., 2012) or that the lack of Repo induces Srp expression non autonomously. Similar results were obtained upon using a second early hemocyte marker, U-shaped (Ush) (**Figure 5C-D'''**): 6.8 +/-0.6 cells/hemisegment show Ush/GFP co-localisation in *sca>gcm;repo* LOF,*repo-nGFP* embryos as compared to 2.8+/-0.7 cells in *sca>gcm;repo-nGFP* embryos (n=10 hemisegments in 3 embryos, ANOVA $p=6.10^{-4}$). Within the neural tissue, we also found Srp or Ush positive cells that are GFP negative (empty arrowheads in **Figure 5B'-B''',D'-D'''**). These cells likely represent hemocytes that have moved into a neural tissue that is no longer properly formed/insulated (Shklyar et al., 2014).

Second, we found that Repo is sufficient to repress the expression of hemocyte genes in endogenous glia. We introduced the *srp(hemo)>CD8GFP* transgene in *repo* LOF, *repo-nRFP* animals and found GFP expression (hemocyte tracer) in a fraction of RFP positive cells (glial tracer) in the *repo* LOF embryos (**Figure 6A-C''**). This does not occur in control animals and is in agreement with the finding that Repo represses the expression of the *srp(hemo)* promoter in S2 cells (**Figure 3I**). Because the GFP of the *srp(hemo)>GFP* line is localized in the membrane and the RFP of the *repo-nRFP* line in the nuclei, we could not formally exclude the possibility that the co-localization indicated the presence of hemocytes within the mutant nervous system and engulfing the presumptive glia. We hence used the anti-Srp antibody and again found expression of the hemocyte marker in presumptive glial cells (*repo* LOF, *repo-nGFP*) **(Figure 6E,F)**. In

similar assays, we found nuclear co-localization between Ush labeling and GFP (**Figure 6G,H**). In total, 11,6 % of the presumptive glia (GFP positive cells) express Srp (2.2 +/-0.8 cells per hemisegment are double positive GFP/Srp, n=10 hemisegments in 3 embryos, W p= 0.0105) and 26 % express Ush ectopically (4.9 +/-0.5 cells per hemisegment are double positive GFP/Ush, n=3 hemisegments in 3 embryos, W p= 0.009). This reveals for the first time a hematopoietic potential for *Drosophila* embryonic glial cells.

In addition, we analyzed the expression of another hemocyte marker by labelling the *repo LOF; repo-nGFP* embryos with the Singed antibody. *singed* (*sn*) codes for a Fascin ortholog that is crucial for hemocyte migration (Zanet et al., 2009) and the antibody strongly labels the embryonic hemocytes (**Figure 6I-L''**). The *repo* LOF embryos show Sn labeling in 6% of the GFP positive cells. (**Figure 6I-L''**). As in the assays performed on ectopic glia, we also found Sn expressing cells that corresponds to hemocytes migrating into the defective nervous system (Sn positive/GFP negative cells, **Figure 6M-N'**).

Finally, we asked whether the lack of Repo converts glial cells into mature and functional hemocytes by monitoring the expression of the hemocyte-specific phagocytosis receptor Crq (Franc et al., 1999), but found no ectopic expression of that protein (**Figure 6O,P**), in agreement with the hypothesis that the lack of Repo does not simply reveal a default hemocyte fate. Thus, the lack of the Repo transcription factor triggers the epxression of subsets of hemocyte markers in a fraction of presumptive glia. This could mean that Repo is not sufficient to repress a hemocyte fate in all glial cells or that distinct glial subtypes express different hemocyte markers in the *repo* LOF embryos. To discriminate between the two hypotheses, we followed the approach described by Sklyar *et al.* (Shklyar et al., 2014) and subdivided the ventral nerve cord in two parts along the Z axis: the ventral part mainly contains cortex glial cells, the dorsal part mainly contains axon-associated glial cells (Ito et al., 1995) (**Figure 6D**). The presumptive glia ectopically expressing

the hemocyte transcription factors Srp or Ush are only located dorsally and they correspond to the axon-associated glia. This was confirmed by using anti-Fas2, which recognizes the three dorsally located longitudinal axonal fascicles of the ventral cord (Santos et al., 2007) (**Figure 6E,F**) or a second neuronal marker, anti-HRP (**Figure 6G,H**). On the other hand, the cells that express Sn are located at the position of the cortex glia and are mostly located ventrally **(Figure 6I-L'')**. This phenotype matches the observation that cortex glia are more motile in *repo* mutant embryos (Shklyar et al., 2014).

In sum, Repo represses the expression of distinct hemocyte markers in specific glial subtypes, hence revealing the complexity of this cell population.

Repo acts as the guardian of the glial fate

The fact that only a fraction of the presumptive glia expresses any hemocyte marker in *repo* LOF embryos prompted us to ask whether these cells display other defects. Since Gcm represses the neuronal fate and gain of function experiments suggest that Repo contributes to the process (Yuasa et al., 2003), we explored the possibility that glial cells lacking Repo express neuronal features. We indeed found that a fraction of the presumptive glial cells (22 %) express the pan-neuronal marker Elav (Yao and White, 1991; Berger et al., 2007) in *repo* LOF; *repo-nGFP* embryos (**Figure 7A-D''',F)**. These cells are scattered throughout the ventral nerve cord (**Figure 7B,D**) and do not co-express the hemocyte markers Srp (**Figure 7E-E'''**) or Ush (**data not shown**).

We hence ypothesized that the neuronal and the hemocyte transcriptional programs may compete with each other and asked whether hemocyte markers are ectopically expressed in the ventral cord of embryos lacking Elav, a key factor for neuronal differentiation. No mutant phenotype was observed in these embryos **(last column in Figure 7F,G)**. Interestingly, however, *elav;repo* LOF double mutant embryos that also carry the *repo-nGFP* transgene show twice as

306 many cells expressing the Srp hemocyte marker in presumptive glia as compared to those observed
307 in *repo* LOF embryos (23 % vs. 11% (**3rd and 2nd columns, respectively, in Figure 7F,G)**. Thus,
308 the glial factor Repo contributes to repress the neuronal as well as the hemocyte fates and the
309 neuronal factor Elav contributes to repress the hemocyte fate.

310 To further our understanding on the role of the Repo transcription factor on the glial fate,
311 we also scored the total number of presumptive glia and assessed their proliferative and cell death
312 profile in *repo* LOF embryos. The number of nuclei expressing the GFP in *repo LOF; repo-nGFP*
313 embryos is 30 % lower compared to that observed in wild-type animals (345.8+/-6.9 per embryo
314 in WT compared to 196.0+/-35.8 in *repo LOF*, n=3 embryos, ANOVA p=0.0383). This is in
315 agreement with a slight reduction in cell division and a slight increase in apoptosis: anti-PH3 (Juan
316 et al., 1998) was used to score for glial cell division: 4.9+/-1.0 dividing cells are present per 6
317 hemisegments in WT embryos compared to 0.3+/-0.3 in *repo LOF* (n=3 embryos, W p=0.0361).
318 Apoptosis was scored using the anti-CM1 antibody that recognizes the activated Caspase-3 (**Figure**
319 **8A-B'''**). No cells were observed in WT compared to 10.6+/-1.2 dying cells in *repo LOF* (n=3
320 embryos, 6 hemisegments were counted per embryo, W p=0.0318). It is therefore likely that some
321 cells missing the Repo protein no longer acquire/maintain the right identity and eventually die. To
322 make sure that the co-localization between the presumptive glia (nuclear GFP) and the death maker
323 CM1 identifies dying cells (**Figure 8A-B''',E**), rather than glial cells that are phagocytosing dead
324 bodies, we compared the results obtained on *repo LOF; repo-nGFP* with those obtained on *repo;*
325 *repo-CD8GFP* embryos, in which the GFP is tagged to the membrane (**Figure 8E**).

326 As expected, in the latter case we did not observe co-localization between the GFP and
327 CM1 (**Figure 8C-C'''**). Moreover, this data further confirmed the lack of phagocytosis observed
328 in *repo LOF* embryos **(Figure 8D)**, likely due to defective SIMU and Draper expression (Shklyar

et al., 2014). Indeed, while in wild-type embryos glial cell membranes completely enwrap apoptotic bodies (**Figure 8C-C'''**), in *repo* LOF embryos these contacts are no longer established.

In sum, Repo acts as a true guardian of the glial fate, in line with the fact that it is the only transcription factor that is expressed in all glia and only in glia.

Discussion

During development, pioneer transcription factors trigger specific cell fates. More and more data however show that these factors act in multiple lineages, raising the question of how does each lineage differentiate at the right place. Here we show that a pioneer factor acts in concert with tissue-specific cues to trigger distinct fates in different territories and that this distinction is maintained through reinforcing inhibitory pathways. The *Drosophila* Gcm zinc finger protein promotes hematopoiesis in the procephalic mesoderm and gliogenesis in the nervous system. The expression of its target and pan-glial transcription factor Repo is repressed in the hematopoietic anlagen by mesodermal cues. In turn, Repo represses the expression of hemocyte genes. These sequential regulatory steps explain how Gcm induces two functionally related but alternative cell fates in different territories.

Tissue-specific cues inhibit the gliogenic potential of Gcm in the hematopoietic anlagen

The *Drosophila* transcription factor Gcm is expressed and required for the differentiation of glia and blood, which share immune features but also perform specific functions in the immune and nervous systems. These cells originate from different layers, glia from the ectoderm, hemocytes from the mesoderm, and therefore display distinct molecular landscapes. We here show that the

mesoderm-specific transcription factor Twi and its target *miR*-1 repress the expression of the pan-glial gene Repo in the hemocyte anlagen. Thus, the mesodermal molecular landscape controls Gcm activity and biases its transcriptional output towards hemocyte differentiation.

The coordinated activity of pioneer and tissue-specific factors allows a limited number of transcription factors to produce the high diversity of cell types present in complex organisms. For example, the vertebrate GATA transcription factors regulate the development of hematopoietic, neural, cardiac or reproductive tissues (Cantor and Orkin, 2005; Zaytouni et al., 2011; Chlon and Crispino, 2012) and control specific target genes in the different tissues due to the activity of tissue-specific transcription factors that modify the transcriptional output of the GATA factors (Cantor and Orkin, 2005). It will be interesting whether in that case as well post transcriptional regulation contributes to the acquisition of cell specificity.

The Repo homeodomain containing factor locks cells in the glial fate

Gcm is expressed and necessary at early stages of glial development, whereas the homeodomain containing Repo protein is stably expressed in the glial cells. The lack of late glial markers observed in *repo* mutant embryos initially suggested a role of Repo in glial terminal differentiation (Xiong et al., 1994; Yuasa et al., 2003). However, the ectopic expression of non-glial markers in those embryos shows that Repo also controls cell plasticity. This shows that homeodomain containing transcription factors can provide the molecular relay from multipotency to a fully differentiated state once the transient expression of pioneer factors extinguishes.

The robustness of the glia and hemocyte fates relies on the activity of cell-specific genes: Repo as well as Elav repress the expression of Srp in the nervous system, whereas Twi/*miR-1* repress the expression of Repo in the mesoderm. Moreover, Srp and Gcm co-expression in the

mesoderm also repress Repo expression (**data not shown**). These inhibitory interactions ensure that the glial and the hemocyte fates are mutually exclusive.

Our data also suggest that glial (Repo) and neuronal (Elav) factors both repress ectopic hematopoiesis in the neural territory while counteracting each other to maintain the glial and the neuronal fates, respectively. This molecular network explains why cells adopt the neuronal default fate in the absence of Gcm whereas they start expressing hemocyte markers in the absence of Repo, and even more so in the absence of both Repo and Elav.

Thus, cell-specific pathways and feedback loops allow a single pioneer factor to affect different cell fates. Such molecular checkpoints acting in parallel and in sequence allow the maintenance of a stable fate.

Lack of Repo triggers different phenotypes in distinct glial subtypes

The glial cells of the embryonic ventral nerve cord are subdivided into three main subtypes (surface, cortex and axon-associated) based on their morphology, position and function (Ito et al., 1995; Beckervordersandforth et al., 2008). The large and flattened glial cells associated to the surface form the BBB (Auld et al., 1995). Glial cells located in the cortex are star-shaped and intermingled with neuronal bodies, their cytoplasmic projections contacting multiple synapses (Freeman and Doherty, 2006; Freeman, 2015). Cortex glia help clearing the debris induced by neuronal programmed cell death (Freeman et al., 2003; Shklyar et al., 2013; Shklyar et al., 2014) (Kurant et al., 2008). Finally, glial cells associated to the axons enwrap them in a multi-layer sheath promoting the conduction of nerve impulses and a subset of them has also been called astrocyte-like glia (Hidalgo and Booth, 2000; Sepp et al., 2000; Sepp and Auld, 2003; Freeman and Doherty, 2006; Freeman, 2015). These glia are known to act as scavengers in response to developmental signals and to trauma, likely due to their proximity to signaling axons. Typically, in the adult brain

399 they phagocyte degenerating axons after brain injury (Doherty et al., 2009) and, after puparium
400 formation, axon-associated glia of the mushroom body control ecdysone-dependent axons pruning
401 (Awasaki and Ito, 2004; Kato et al., 2011; Kato and Hidalgo, 2013; Boulanger and Dura, 2014;
402 Hakim et al., 2014).

403 Repo is expressed in the three cell types and its lack affects them all (Giesen et al., 1997;
404 Yuasa et al., 2003; Kerr et al., 2014), however the *repo* mutant phenotypes reveal the underlying
405 diversity of the glial subtypes as, in the absence of Repo, axon-associated glia express early
406 hemocyte transcription factors but not Sn, whereas cortex glia express Sn, but not the Srp or Ush
407 transcription factors. Of note, Sn is necessary for cell motility (Adams, 2004; Zanet et al., 2009)
408 and Kurant and collaborators (Shklyar et al., 2014) observed that *repo* mutant cortex glia are very
409 motile. In the future, it will be interesting to determine the transcriptional landscape of the different
410 glial subtypes as, for example, cortex glia may be specialized in removing dead cell bodies whereas
411 axon-associated glia may specifically target and remove axons and dendrites.

412 Finally, our data strongly suggest that, although glial cells act as macrophages, they do not
413 have a default hemocyte phenotype, rather, they constitute a very specialized population of
414 scavenger cells. Similarly, vertebrate microglia, cells of immune origin that provide the first
415 response to nervous system challenge, display a molecular signature that is distinct from that of
416 macrophages (Prinz and Priller, 2014).

417

418 **Of flies and vertebrates…**

419 *Drosophila* and vertebrate glial cells share numerous functions controlling neuron
420 homeostasis, recycling neurotransmitters and insulating axons (Freeman and Doherty, 2006),
421 however the transcriptional program triggering the first steps of gliogenesis are not evolutionarily

conserved. In *Drosophila*, the Gcm transcription factor constitutes the major regulatory gene and acts as a molecular switch between neuron and glial cells. Although the vertebrate Gcm orthologs seem to maintain some gliogenic potential *in vitro* (Kim et al., 1998; Reifegerste et al., 1999; Buzanska et al., 2001; Iwasaki et al., 2003; Soustelle et al., 2007), they are neither expressed nor required in glia. Moreover, no true glial determinant has been so far identified in vertebrates (Hitoshi et al., 2011). Even more strikingly, the vertebrate genomes do not contain the coding sequences for Repo (no orthologs found so far), the only fly transcription factor that is specific to all lateral glia and only to glia, a molecular signature that seems shared throughout the Arthropod clade (Wakamatsu, 2004; Boyan et al., 2011; Mysore et al., 2011; Nasu and Hara, 2012).

Our findings raise the question of the evolutionary link between vertebrate and *Drosophila* gliogenesis (Hartline, 2011). While the hypothesis of an independent origin of vertebrate and invertebrate glia remains to be tested, the comparative analysis of those glia has tremendously improved our understanding of the bases of nervous system regeneration. *Drosophila* glia indeed constitute an excellent model to investigate the mechanisms governing CNS repair following traumatic injury (Leyssen and Hassan, 2007; Kato et al., 2011). In this contest, and in light of recent data showing that mature astrocytes and oligodendrocytes can be reprogrammed into functional neurons to promote CNS regeneration (Heinrich et al., 2010; Guo et al., 2014; Su et al., 2014), it will be interesting to study whether the loss of Repo triggers glial cell conversion into neurons in the adult *Drosophila* injured CNS.

Finally, sequencing the genome and analyzing the single cell transcriptome of simple organisms has become an important tool to understand the molecular and cellular bases of evolution. Future analyses will establish when Gcm and Repo appear in evolution and where are they expressed/required within/outside the nervous system.

Materials and Methods

Fly stocks

Flies were kept at 25 °C. w^{1118} was used as wild-type. *repo-nGFP* was used to drive nuclear GFP expression under the control of the 4.3kb *repo* promoter, which recapitulates the full *repo* expression pattern (Jones, 2005; Laneve et al., 2012). gcm^{34} (Bernardoni et al., 1999) was used as a *gcm* hypomorphic allele carrying a *lacZ* insertion. The *Df(2L)132* (Kammerer and Giangrande, 2001) deletes the entire *gcm* locus and was used as a null allele. $repo^{52}$, $repo^{84}$ (Xiong et al., 1994; Halter et al., 1995), twi^{1} (Castanon et al., 2001) and $elav^{4}$ (Bloomington Center) are null alleles.

The *UAS/Gal4* system was used for cell-specific manipulation of gene expression. *srp(hemo)Gal4* triggers expression in hemocytes (Bruckner et al., 2004), *scaGal4* throughout the neurogenic region (Bloomington stock Center), *twiGal4* (Baylies and Bate, 1996) throughout the mesoderm and *gcmGal4* (Soustelle and Giangrande, 2007) combined to *repo-Gal80* (gift of B. Altenhein) throughout the hemocyte anlagen. Finally, *repoGal4* was used to drive gene expression in glial cells (Lee and Jones, 2005).

The following transgenes were also used: *UAS-CD8GFP* (targeting GFP expression to the membrane), *UAS-RFP* (Bloomington stock Center), *UAS-GFP (*Bloomington stock Center); *UAS-repo* (Yuasa et al., 2003); *UAS-twi* (Baylies and Bate, 1996)*; UAS-gcm(F18A)* **(Figure 5)** (weak Gcm over-expression), *UAS-gcm(RS1)* **(Figure 1C,D,I)** or *UAS-gcm(M24A)* **(Figure 1H, Figure 4)** (medium Gcm over-expression) (Bernardoni et al., 1998). The combination of *UAS-gcm(M24A)* and *UAS-gcm(F18A)* provided a strong Gcm over-expression **(Figure 1H)**.

Immunohistochemistry

Embryo collections were done on plates containing agar, apple juice and yeast. Dechorionated embryos were fixed in 4% formaldehyde in PBS for 20 min, permeabilized with 0.3 % Triton-x100 in PBS (PTX), blocked by 0.5% Blocking Reagent (Roche) in PTX for 1 h and labeled overnight at 4 °C with the following antibodies: rabbit (rb) anti-Repo (1/10), mouse (m) anti-Repo (1/10), m anti-Singed (1/50) and rat anti-Elav (1/200) (DHSB); guinea pig (gp) anti-Repo (1/1000) and gp anti-Nazgul, (1/200) (gift of B. Altenhein) (von Hilchen et al., 2010); mouse (m) anti-Ush (1/1000) (Cubadda et al., 1997); rb anti-Srp (1/1000) (gift of R. Reuter) (Sam et al., 1996; Petersen et al., 1999); m anti-P1 (1/10) (gift of E. Kurucz) (Kurucz et al., 2007); rb anti-Crq (1/500) (gift of J.L Dimarcq and J. Hoffmann) (Franc et al., 1996); m anti-Fas2 (1/100) (gift of C.S. Goodman) (Grenningloh et al., 1991); rb anti-HRP (1/500) and rb anti-β-Gal (1/500) (Cappel) and chicken anti-GFP (1/1000) (Abcam); m anti-β-Gal (1/200) (Sigma); rat anti-RFP (1/100) (chromotek); rb anti-DCP-1 (1/50) (Cell Signaling Technology).

The secondary antibodies were FITC-, Cy3 or Cy5 conjugated (1/400, Jackson). Images were taken with the SP2 or the SP5 Leica confocal microscopes and processed using Fiji (Schindelin et al., 2012).

Srp signal intensity was measured on confocal images acquired with hybrid detector in photon counting mode. The mean gray value measurement tool from Fiji was used to estimate the intensity of the signal (in Arbitrary Unit, AU) from 50 hemocytes in at least 3 embryos (Schindelin et al., 2012).

Co-transfection, Western blot and luciferase assays

Drosophila S2 cells were grown in Schneider medium (Fisher Scientific) complemented with 10% heat inactivated Fetal Calf Serum and 0.5% Penicillin/Streptomycin. 6 x 10^6 cells were

493 cultured in six well culture dish 12 h prior transfection. 5 µg of total plasmid mix were transfected
494 using the Effectene Kit (Qiagen) according to manufacturer's instructions. The *psrp(hemo)Gal4*
495 plasmid provided a *srp* transcriptional reporter (Bruckner et al., 2004) upon co-transfection with
496 the *pUAS-GFP* plasmid. The *pPac5C-repo* plasmid was used to induce Repo expression (Yuasa et
497 al., 2003) and *pPac5C-lacZ* as a transfection control. The *pPac5C* plasmid was used to equilibrate
498 the amount of transfected DNA. Cells were harvested 24 h after transfection in Tris-HCl 25 mM
499 pH 7.9, 400 mM KCl, 10 % glycerol and total proteins were extracted by three freezing-thawing
500 steps. Protein expression was detected from protein lysate according to standard Western blot
501 procedure. The following primary antibodies were used: m anti-β-Gal (1/2500, Sigma), rb anti-
502 GFP (1/5000, Molecular Probes), m anti-Repo (1/20, DHSB). m anti-HRP and rb anti-HRP
503 (1/5000, Jackson ImmunoResearch) were used as secondary antibodies.
504 For the luciferase assay, *Drosophila* S2 cells were cultured in a 24-well plate, in the same
505 conditions as previously described. Plasmid transfections were carried out using Effectene
506 (Qiagen) following manufacturer's instructions. *pMTGal4-GFP, pUAST-Luciferase-Luciferase*
507 *3'UTR, pUAST-Luciferase-Repo 3'UTR*, *pUAST-Luciferase-Repo 3'UTR ΔmiR-1* and *pTK-Renilla*
508 were all used at 20 ng/mL and *pTub-miR-1* was used at 50 ng/mL. The cells were cultured 2 days
509 prior induction with 500 µM of copper sulphate. The luciferase assay was done 18h after induction,
510 using the Dual-Glo Luciferase assay kit (Promega) according to manufacturer's instructions. Three
511 independent transfections were averaged with standard deviation. Statistical significance was
512 calculated with Graphpad Prism software using t-test.
513
514 **RNA extraction, reverse transcription and qPCR**
515 Total RNA was purified from stage 5-11 embryos by TriReagent (MRC). 1 µg of purified
516 RNA was reverse transcribed by SuperScript II reverse transcriptase (Invitrogen) using oligodT

primers (5 µM). mRNAs were analyzed by qPCR using Sybr Green (Roche) Master Mix, the thermocycler Roche LightCycler480 and the following oligonucleotides:

repo forward : 5' AAGCAGCAGCAAGAAGAAGG 3'

repo reverse : 5' ATACGGAGCACGTTCAAAGG 3'

actin5C forward : 5' GCAGCAACTTCTTCGTCACA 3'

actin5C reverse : 5' CTTAGCTCAGCCTCGCCACT 3'

For each gene, the mRNA levels were automatically calculated (LightCycler480 Software, release 1.5.0) by calibration to gene-specific standard curves generated on input cDNAs. Collected values, derived from three amplification reactions, each performed in three independent experiments, were normalized to *actin5C* mRNA amounts.

Statistics

All the experiments were performed in at least three biological replicates. Statistical relevance was assigned by calculating means, standard errors. Whenever the data showed normal distribution **(Figure 1H,I, 3I)**, they were analyzed by the ANOVA test, whenever they did not **(Figure 7F,G)** by Kruskal-Wallis (KW) and Wilcoxon (W) tests. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Author contribution

GT and AG designed the experiments. GT, PC and DM did the experiments. GT, PC, JZ, DM, MF and AG analyzed the data. GT, PC and AG finalized the manuscript.

Acknowledgements

We thank B. Altenhein, K. Brückner, M. Crozatier, L. Waltzer, M. Logan, E. Kurant, R. Reuter, E. Kurucz, J.L Dimarcq, J. Hoffmann, C. Goodman, the DHSB and the Bloomington Stock Center for reagents and flies. We thank all the lab members for comments on the manuscript, C. Diebold, C. Delaporte, M. Pezze, the fly, imaging and antibody facilities for technical assistance and D. Dembele for help with statistics. We thank Alison Brewer for help with Luciferase assays. This work was supported by INSERM, CNRS, UDS, Ligue Régionale contre le Cancer, Hôpital de Strasbourg, ARC and ANR grants. P. Cattenoz was funded by the ANR and by the ARSEP, G Trebuchet by governmental and by ARC fellowships. This work was also supported by grants from the Ataxia UK (2491) and the NC3R (NC/L000199/1) awarded to M.F. The IGBMC was also supported by a French state fund through the ANR labex.

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739 Yao KM, White K (1991) Organizational analysis of elav gene and functional analysis of ELAV protein of
740 Drosophila melanogaster and Drosophila virilis. Mol Cell Biol 11:2994-3000.
741 Yuasa Y, Okabe M, Yoshikawa S, Tabuchi K, Xiong WC, Hiromi Y, Okano H (2003) Drosophila homeodomain
742 protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors.
743 Development 130:2419-2428.
744 Zanet J, Stramer B, Millard T, Martin P, Payre F, Plaza S (2009) Fascin is required for blood cell migration
745 during Drosophila embryogenesis. Development 136:2557-2565.
746 Zaytouni T, Efimenko EE, Tevosian SG (2011) GATA transcription factors in the developing reproductive
747 system. Advances in genetics 76:93-134.
748

749

Table 1

Drivers used to target the neurogenic region, the mesoderm and the procephalic mesoderm.

The 1st column indicates the genotype, the 2nd column indicates the region expressing the driver (embryo at stage 8, lateral view, anterior to the left) and in a cross-section of the embryo (dorsal to the top) and the 3rd column indicates the region targeted.

Figure legends

Figure 1: Twi negatively regulates Repo expression.

A-D) Confocal projections of embryos stage 14 *scaGal4* or *sca>* (Control, **A**), *sca>twi* (*twi* GOF, **B**), *twi>gcm* (*gcm* GOF, **C**) and *twi>gcm;twi-/+* (*gcm* GOF, *twist* het, D) immunolabeled for the glial marker Repo (blue). Ventral view. Unless otherwise specified, all scale bars represent 100 µm and anterior is to the left. (**E-F''**) Confocal projections of wild-type (**E**) and *twi-/-* (*twi* LOF, **F**) embryos labeled for the Repo glial marker (blue) and for the hemocyte Srp marker (red). Lateral view. (**F'**) and (**F''**) represent a single section of the inset indicated in (**F**), they show Srp labeling only and co-labeling with Repo, respectively. The white arrowheads indicate cells expressing Srp and Repo. (**G**) Confocal projections of *gcmGal4,repoGal80/+;UAS-CD8GFP* (*gcm(hemo)>GFP*) embryos labeled for Repo (blue) and GFP (green). Lateral view (upper panel) and ventral view (lower panel). The region defined by the dashed line indicates the Central Nervous System (CNS). Note that GFP expression is excluded from glia. (**H,I**) Relative quantification of *repo* mRNA by qPCR from stage 5-11 embryos of the following genotypes: *gcm(hemo)>* (Control) and *gcm(hemo)> gcm* GOF (Weak, Medium and Strong *gcm* GOF) in (**H**); *gcm(hemo)>* (Control), *gcm(hemo)>medium gcm* (*Med. gcm* GOF) and *gcm(hemo)>medium gcm + twi* (*Med. gcm* GOF,

twi GOF) in (**I**). *gcm* levels are relative to *actin* levels, n indicates the number of independent assays, see the Experimental Procedure section for the statistic tests. (**J-L**) Confocal projections of embryonic ventral cords of the following genotypes: gcm^{34}/+ (**J**), gcm^{34}/gcm^{34} (**K**) and $gcm^{34}/Df(2L)132$ (**L**). Labeling: β-Gal (green), Srp (red) and the neuronal marker Elav (gray). The gcm^{34} line represents a P element partial excision that retains the LacZ gene, allowing monitoring of *gcm* expression. β-Gal/Srp double positive cells (yellow, asterisks) are located outside the ventral cord (dashed line) and label the circulating hemocytes.

Figure 2: *miR-1* prevents Repo expression in the hemocyte lineage.

(A) Schematic representation of the *repo* locus in the *Drosophila* genome (dm3). UTRs and coding exons are indicated by plain blue boxes (thin and thick, respectively) and the intron by a blue line. The two putative *miR-1* binding sites in the *repo* 3'UTR are indicated. (**B,C**) Confocal projections of embryos of the following genotypes: wild-type and *miR-1* LOF (-/-), lateral view, stage 14, labeled for Repo (blue) and Srp (red). (**C'**) and (**C''**) represent a single section of the inset indicated in (**C**), they show Srp labeling only and co-labeling with Repo, respectively. (**D**) Number of hemocytes expressing Srp and Repo in wild-type and in *miR-1* mutant embryos (-/+ and -/-). n indicates the number of embryos analyzed for each genotype. (**E**) Schematic representation of the three Luciferase reporter vectors that were used in the co-transfection assays: the top one is the Control vector carrying the Firefly Luciferase coding sequence and the Firefly 3'UTR under the UAS promoter. In the second construct (middle), the 3'UTR has been replaced by the *repo* 3'UTR and in the last construct (bottom), the two *miR-1* binding sites of the *repo* 3'UTR have been mutated. (**F**) Quantification of the Luciferase activity in extracts from S2 cells co-transfected with *pTub-miR-1*, *pTK-Renilla* and either *pUAST-Luciferase-Luciferase-3'UTR* (*Firefly* 3'UTR, gray),

pUAST-Luciferase-Repo-3'UTR (*repo* 3'UTR, green) or *pUAST-Luciferase-Repo-3'UTRΔmiR-1* (*repo* 3'UTR *ΔmiR-1*, red), the values are normalized with the Renilla activity.

Figure 3: Repo can repress hemocyte differentiation.

(A-H'') Embryos *gcm(hemo)>CD8GFP* (Control) or *gcm(hemo)>CD8GFP,repo* (*repo* GOF). **(A,B)** represent confocal projections of embryos labeled for GFP (green), Srp (red) and Repo (blue), dorsal view, stage 16, the empty arrowheads indicate the Srp positive GFP negative cells of the fat body. **(A')** and **(B')** show the Srp signal alone. (**A'',A''',B'',B'''**) show single sections of the insets indicated in (**A,B**), the arrowheads indicate the hemocytes (GFP/Srp double positive cells). Note that Repo is expressed in GFP positive cells in *repo* GOF (**B''**) and that the levels of Srp upon Repo overexpression (**B'''**) are much lower compared to those observed in the wild-type embryo (**A'''**). (**C,D**) represent confocal projections of embryos labeled for the hemocyte marker P1 (red), dorsal view, stage 14. (**E-F**) represent confocal projections of embryos labeled for GFP (green) and the glial marker Nazgul (red), lateral view, stage 14, (**E',F'**) show the Nazgul signal alone. (**G,H**) represent single confocal sections of embryos labeled for DAPI (blue), CD8GFP (green) and the apoptotic marker DCP-1 (gray). (**G',G'',H',H''**) show the insets indicated in (**G,H**), the arrowheads indicate cells double positive for CD8GFP and DCP-1. (**I**) Western blot on protein extracts from S2 cells co-transfected with *psrp(hemo)Gal4*, *pUAST-GFP* and increasing amounts of *pPac5C-repo* (0 to 3 µg). *pPac5C-lacZ* was used as a transfection control. The histogram represents GFP/β-Gal relative quantification. The amounts of transfected Repo were also verified. n indicates the number of co-transfection assays. (**J-M**) Embryos *srp(hemo)>RFP* (Control) or *srp(hemo)>RFP,repo* (*repo* GOF), lateral view, stage 14. (**J,K**) represent confocal projections of embryos labeled for RFP (red) and Repo (green). (**J'**,**K'**) show the Srp signal alone

from (**J,K**). (**J'',K''**) represent single sections of the insets indicated in (**J,K**). (**L,M**) represent confocal projections of embryos labeled for Nazgul (red).

Figure 4: Gcm has a strong gliogenic potential in hemocyte precursors.

(A-D''') Embryos *gcm(hemo)>CD8GFP* (Control, **A,A'C,C'**) and *gcm(hemo)>CD8GFP,medium gcm* (Medium *gcm* GOF, **B,B'D,D'**). **(A-B')** represent confocal projections of embryos labeled for GFP (green) and Repo (blue), lateral view, stage 14. **(B'',B''')** represent single confocal sections of the inset indicated in **(B')**, the arrowheads indicate cells double positive for CD8GFP and Repo. **(C-D')** represent confocal projections of embryos labeled for GFP (green) and Nazgul (red), lateral view, stage 16. Brackets indicate territories exhibiting hemocytes. Note that the yellow color observed in (**C'**, oval) is an artifact created by the projection. **(D'',D''')** represent single confocal sections of the inset indicated in **(D')**, arrowheads indicate ectopic glial labeling in hemocytes over-expressing Gcm. **(E-F''')** Single confocal sections of medium *gcm* GOF (*gcm(hemo)>CD8GFP,medium gcm*) embryos labeled for Srp (red), Repo (blue) and GFP **(E-E''')** and Crq (red), Repo (blue) and GFP **(F-F''')**. Hemocytes are indicated by asterisks, those that also express Repo by arrowheads. Note that Repo ectopic expression does not affect Srp or Crq expression. Scale bars in **(E,F)**: 50 µm.

Figure 5: Repo represses the Gcm hematopoietic potential in the neuroectoderm.

(A-D) Confocal projections of embryos *sca>weak gcm;repo-nGFP* (Weak *gcm* GOF, **A,C**) and *sca>weak gcm/repo-nGFP;repo-/-* (weak *gcm* GOF, *repo* LOF, **B,D**) labeled for GFP (green) and Srp (red) **(A-B''')** or GFP (green) and Ush (red) **(C-D''')**, ventral view, stage 16. The dashed line indicates the ventral nerve cord (VNC) **(A-D)**. **(B'-B''', D'-D''')** represent single sections of the insets indicated in **(B, D)**, they show nGFP labeling only, Srp or Ush labeling only and co-labeling

Srp or Ush with nGFP, respectively. White arrowheads indicate nGFP/Srp **(B'-B''')** or nGFP/Ush **(D'-D''')** double positive cells, empty arrowheads indicate Srp or Ush positive and nGFP negative cells in *gcm* GOF *repo* LOF embryos. These are hemocytes recruited to the VNC that is not properly insulated due to the mutant background (Shklyar et al., 2014).

Figure 6: Repo is required to repress hemocyte transcription factors in developing glia.

(**A,B**) Confocal projections of embryos *srp(hemo)>CD8GFP/repo-nRFP* (Control, **A**) and *srp(hemo)>CD8GFP/repo-nRFP;repo-/-* (*repo* LOF, **B**) labeled for GFP (green) and RFP (red), ventral view. (**B'-B''', C-C''**) show single sections of the insets indicated in (**B**). Note that the single sections were acquired at different focal planes in the VNC. The arrows indicate GFP/RFP double positive cells. (**D**) Schematic representation of a transversal section of the VNC from a mature embryo. Glial cell subtypes are defined according to their localization: surface pale (blue), cortex (red) and axon-associated glia (green) (Ito et al., 1995; Beckervordersandforth et al., 2008). (**E-P**) Embryos of the following genotypes: *repo-nGFP* (Control) and *repo-/-;repo-nGFP* (*repo* LOF). The analyses were performed upon subdividing the VNC in a ventral and in a dorsal part, according to the schematic shown in (**D**), the position of the section along the dorso/ventral axis of the VNC is indicated on the left side of the panels. Scale bar in **(E-P)**: 50 µm. Stage 15 embryos are labeled for GFP (green), Fas2 (gray) and Srp (red) (**E,F**); GFP (green), HRP (gray) and Ush (red) (**G,H**). Stage 14 embryos are labeled for GFP (green) and Sn (red) (**I-L''**), (**L',L''**) show single sections of the inset indicated in (**L**). (**M,N**) Confocal projections of the whole VNC labeled for GFP (green), Sn (red) and Elav (gray), the dash line indicates the position of the z-axis reconstitution of the VNC presented in (**M',N'**). Note the presence of Sn positive/GFP negative cells within the VNC in *repo* LOF embryo; these are hemocytes recruited to the VNC following the loss of *repo* (Shklyar et al., 2014). (**O,P**) Embryos labeled for GFP (green) and Crq (red).

Figure 7: Repo represses both hemocyte and neuronal differentiation

(**A-D’’’**) Embryos of the following genotypes: *repo-nGFP* (Control) and *repo-/-;repo-nGFP* (*repo* LOF), ventral view, stage 15. The ventral and the dorsal parts of the VNC were analyzed separately. Labeling: GFP (green) and Elav (gray). (**B’-B’’’,D’-D’’’**) show single sections of the insets indicated in (**B, D**). Arrowheads indicate ectopic GFP/Elav double positive cells. (**E-E’’’**) Dorsal part of a *repo-/-;repo-nGFP* (*repo* LOF) embryo labeled for Srp (red), Elav (gray) and GFP (green), the channels are presented individually in (**E’**), (**E’’**) and (**E’’’**), respectively. White arrowheads indicate GFP/Elav double positive cells and empty arrowheads indicate GFP/Srp double positive cells. Scale bars in **(A,E)**: 50µm. (**F,G**) Graphs showing the number and the percentage of GFP/Elav double positive cells (**F**) or GFP/Srp double positive cells (**G**) per hemisegment in Control, *repo* LOF, *repo* LOF *elav* LOF double mutant and *elav* LOF embryos. n indicates the number of hemisegments counted in 3 embryos.

Figure 8: *repo* -/- glia undergo apoptosis.

(A-D) Embryos of the following genotypes: *repo-nGFP* (Control, **A**) and *repo-/-;repo-nGFP* (*repo* LOF, **B**) express nuclear GFP. *repo-CD8GFP* (Control, **C**) and *repo-/-;repo-CD8GFP* (*repo* LOF, **D**) express GFP at the membrane, ventral view, stage 15. Labeling: GFP (green) and the apoptotic marker CM1 (red). (**B’-B’’’,C’-C’’’**) show single sections of the insets indicated in (**B, C**). Arrowheads in **(B’-B’’’)** indicate glial cells undergoing apoptosis (co-localisation of nuclear GFP and CM1), whereas arrowheads in **(C’-C’’’)** indicate glial cells enwrapping apoptotic bodies (CD8GFP surrounding CM1 labeled bodies). **(E)** Schematic representation of the GFP/CM1 co-labelling in apoptotic cells expressing nuclear GFP and in phagocytic cells expressing GFP at the membrane.

Table 1

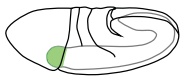
Driver	Expression profile in embryo (stage 8, lateral and cross-section)	Region
scaGal4 (sca>)		Ventral neurogenic region
twiGal4 (twi>)		Mesoderm Mesectoderm
gcmGal4,repoGal80 (gcm(hemo)>) *srp(hemoGal4) (srp(hemo)>)*		Procephalic mesoderm

Table 1
Drivers used to target the neurogenic region, the mesoderm and the procephalic mesoderm. The 1st column indicates the genotype, the 2nd column indicates the region expressing the driver (embryo at stage 8, lateral view, anterior to the left) and in a cross-section of the embryo (dorsal to the top) and the 3rd column indicates the region targeted.

Figure 1

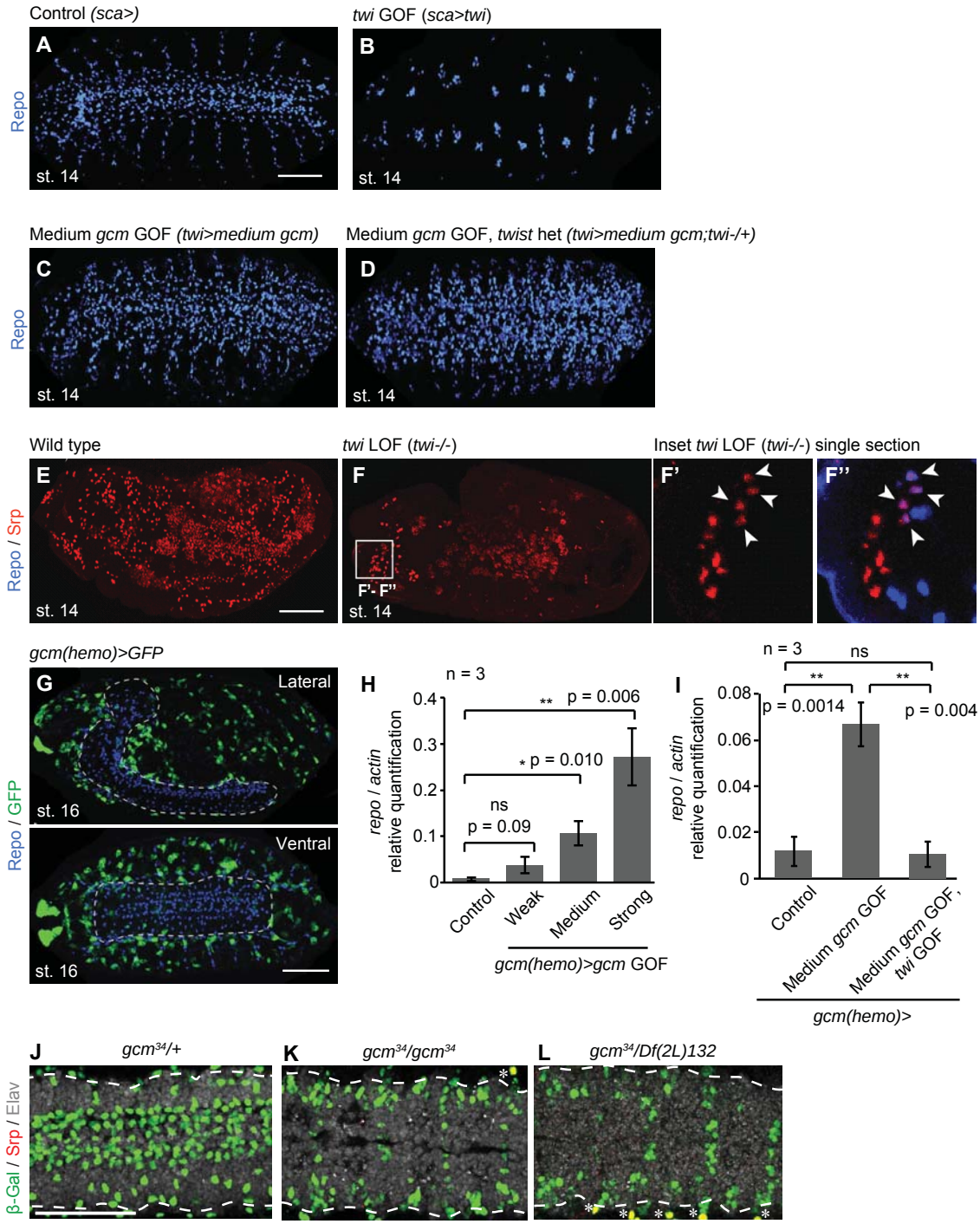

Control (sca>)
twi GOF (sca>twi)
A
B
Repo
st. 14
st. 14
Medium gcm GOF (twi>medium gcm)
Medium gcm GOF, twist het (twi>medium gcm;twi-/+)
C
D
Repo
st. 14
st. 14
Wild type
twi LOF (twi-/-)
Inset twi LOF (twi-/-) single section
E
F
F'
F''
Repo / Srp
st. 14
F'- F''
st. 14
gcm(hemo)>GFP
G
Lateral
st. 16
Ventral
st. 16
Repo / GFP
H
n = 3
repo / actin relative quantification
0.4
0.3
0.2
0.1
0
** p = 0.006
* p = 0.010
ns
p = 0.09
Control
Weak
Medium
Strong
gcm(hemo)>gcm GOF
I
n = 3
ns
**
**
0.08
0.06
0.04
0.02
0
p = 0.0014
p = 0.004
repo / actin relative quantification
Control
Medium gcm GOF
Medium gcm GOF, twi GOF
gcm(hemo)>
J
gcm34/+
K
gcm34/gcm34
L
gcm34/Df(2L)132
β-Gal / Srp / Elav

Figure 2

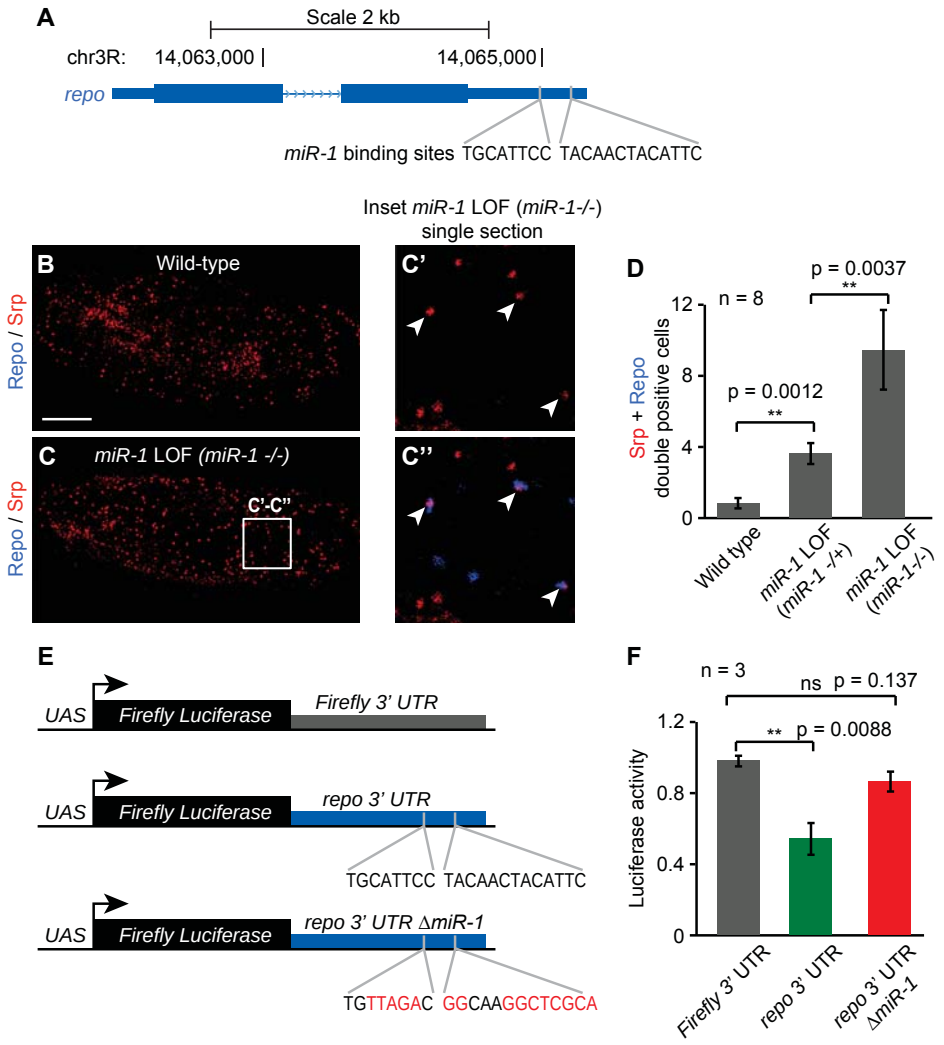

A
Scale 2 kb
chr3R: 14,063,000 | 14,065,000 |
repo
miR-1 binding sites TGCATTCC TACAACTACATTC
Inset miR-1 LOF (miR-1-/-)
single section
B
Wild-type
C
miR-1 LOF (miR-1 -/-)
C'-C''
C'
C''
Repo / Srp
Repo / Srp
D
n = 8
p = 0.0037
**
p = 0.0012
**
Srp + Repo
double positive cells
12
8
4
0
Wild type
miR-1 LOF (miR-1 -/+)
miR-1 LOF (miR-1-/-)
E
UAS
Firefly Luciferase
Firefly 3' UTR
UAS
Firefly Luciferase
repo 3' UTR
TGCATTCC TACAACTACATTC
UAS
Firefly Luciferase
repo 3' UTR ΔmiR-1
TGTTAGAC GGCAAGGCTCGCA
F
n = 3
ns p = 0.137
** p = 0.0088
Luciferase activity
1.2
0.8
0.4
0
Firefly 3' UTR
repo 3' UTR
repo 3' UTR ΔmiR-1

Figure 3

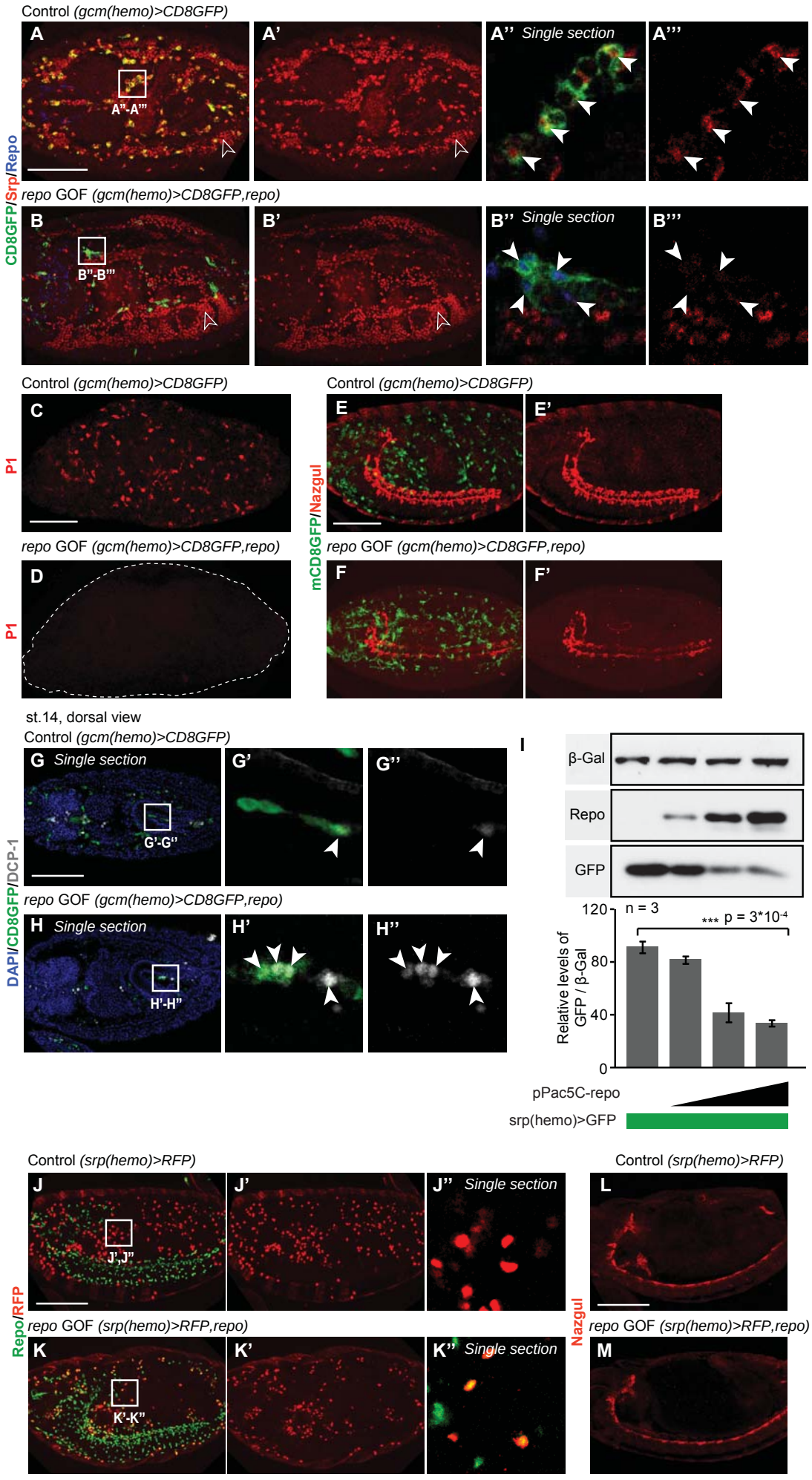

Control (gcm(hemo)>CD8GFP)
A
A'
A'' Single section
A'''
A''-A'''
CD8GFP/Srp/Repo
repo GOF (gcm(hemo)>CD8GFP,repo)
B
B'
B'' Single section
B'''
B''-B'''
Control (gcm(hemo)>CD8GFP)
C
P1
Control (gcm(hemo)>CD8GFP)
E
E'
mCD8GFP/Nazgul
repo GOF (gcm(hemo)>CD8GFP,repo)
D
P1
repo GOF (gcm(hemo)>CD8GFP,repo)
F
F'
st.14, dorsal view
Control (gcm(hemo)>CD8GFP)
G Single section
G'
G''
G'-G''
DAPI/CD8GFP/DCP-1
repo GOF (gcm(hemo)>CD8GFP,repo)
H Single section
H'
H''
H'-H''
I
β-Gal
Repo
GFP
n = 3
*** p = 3*10-4
Relative levels of GFP / β-Gal
120
80
40
0
pPac5C-repo
srp(hemo)>GFP
Control (srp(hemo)>RFP)
J
J'
J'' Single section
J',J''
Repo/RFP
repo GOF (srp(hemo)>RFP,repo)
K
K'
K'' Single section
K'-K''
Control (srp(hemo)>RFP)
L
Nazgul
repo GOF (srp(hemo)>RFP,repo)
M

Figure 4

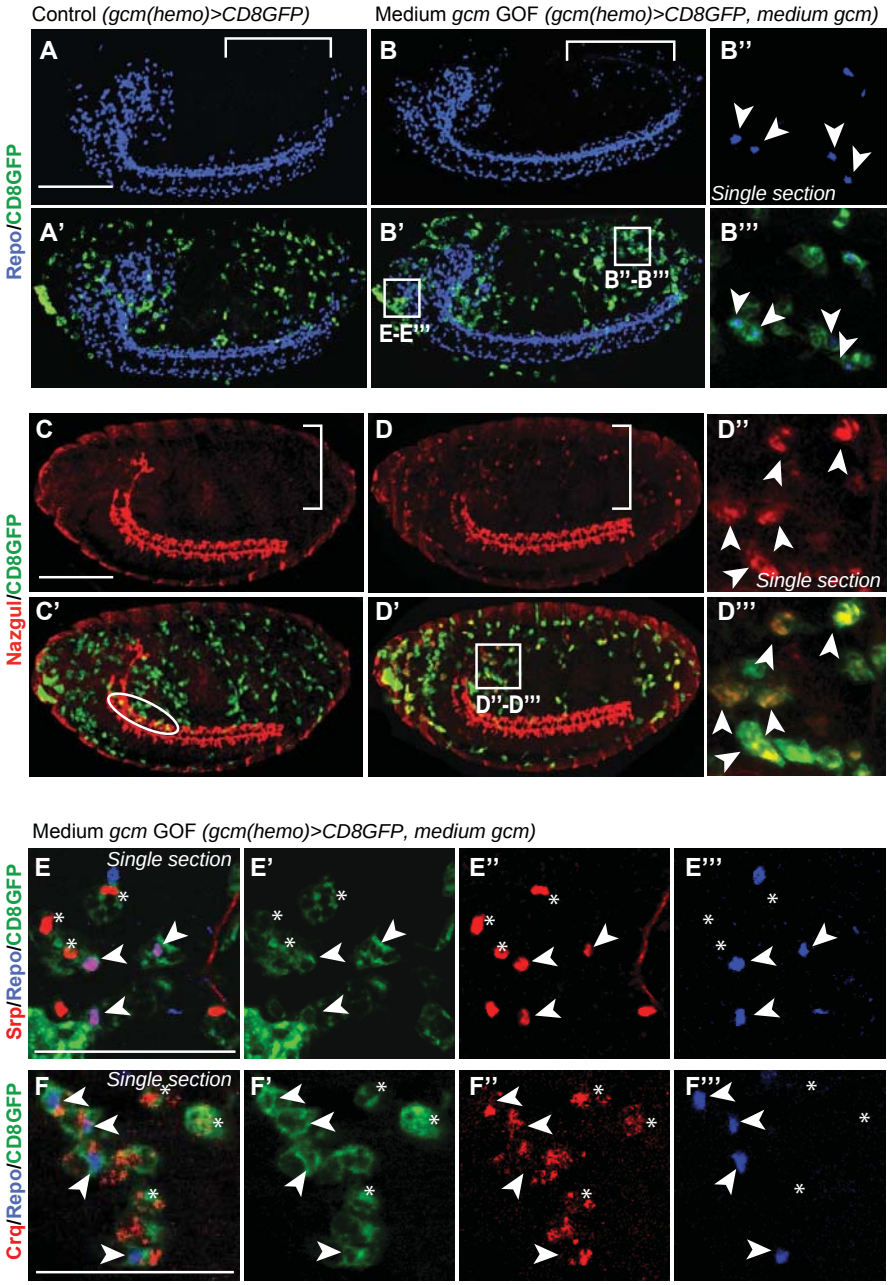

Control (gcm(hemo)>CD8GFP)
Medium gcm GOF (gcm(hemo)>CD8GFP, medium gcm)
Repo/CD8GFP
A
B
B''
Single section
A'
B'
B''-B'''
E-E'''
B'''
Nazgul/CD8GFP
C
D
D''
Single section
C'
D'
D''-D'''
D'''
Medium gcm GOF (gcm(hemo)>CD8GFP, medium gcm)
Srp/Repo/CD8GFP
E
Single section
E'
E''
E'''
Crq/Repo/CD8GFP
F
Single section
F'
F''
F'''

Figure 5

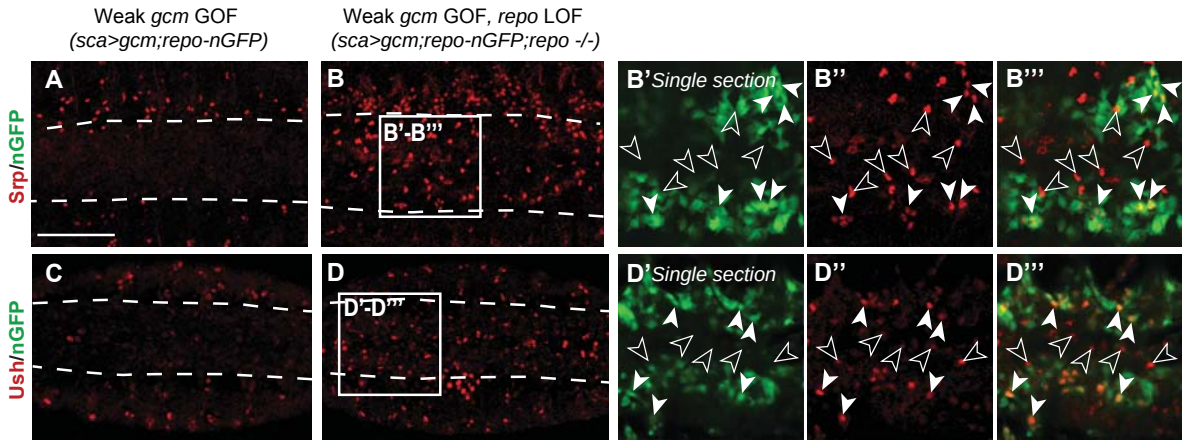

Weak gcm GOF
(sca>gcm;repo-nGFP)
Weak gcm GOF, repo LOF
(sca>gcm;repo-nGFP;repo -/-)
Srp/nGFP
Ush/nGFP
A
B
B'-B'''
B' Single section
B''
B'''
C
D
D'-D'''
D' Single section
D''
D'''

Figure 6

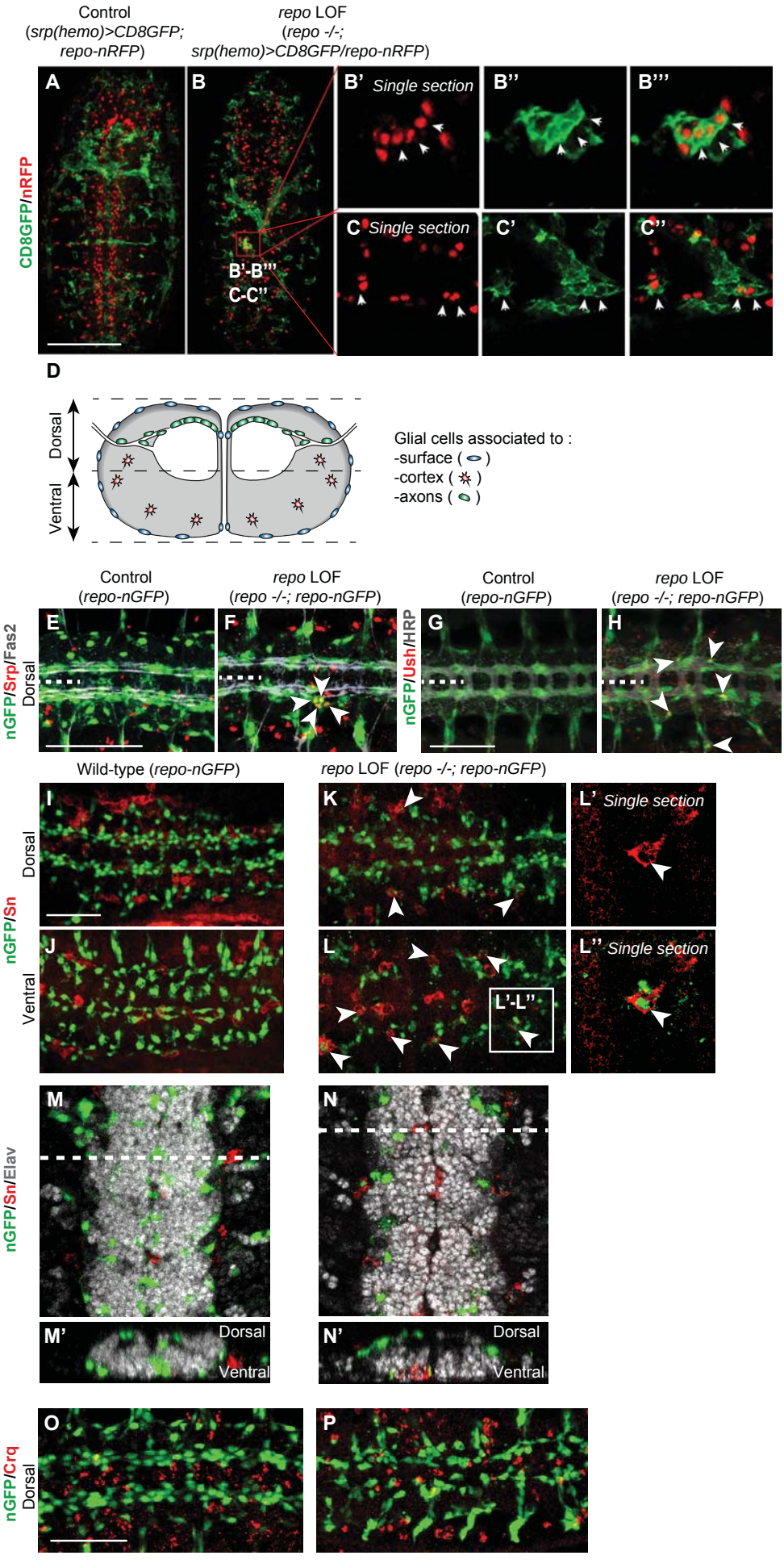

Control (srp(hemo)>CD8GFP; repo-nRFP)
repo LOF (repo -/-; srp(hemo)>CD8GFP/repo-nRFP)
A
B
B' Single section
B''
B'''
C Single section
C'
C''
CD8GFP/nRFP
B'-B'''
C-C''
D
Dorsal
Ventral
Glial cells associated to :
-surface ( )
-cortex ( )
-axons ( )
Control (repo-nGFP)
repo LOF (repo -/-; repo-nGFP)
Control (repo-nGFP)
repo LOF (repo -/-; repo-nGFP)
E
F
G
H
nGFP/Srp/Fas2
Dorsal
nGFP/Ush/HRP
Wild-type (repo-nGFP)
repo LOF (repo -/-; repo-nGFP)
I
J
K
L
L' Single section
L'' Single section
L'-L''
Dorsal
Ventral
nGFP/Sn
M
N
M'
N'
Dorsal
Ventral
nGFP/Sn/Elav
O
P
nGFP/Crq
Dorsal

Figure 7

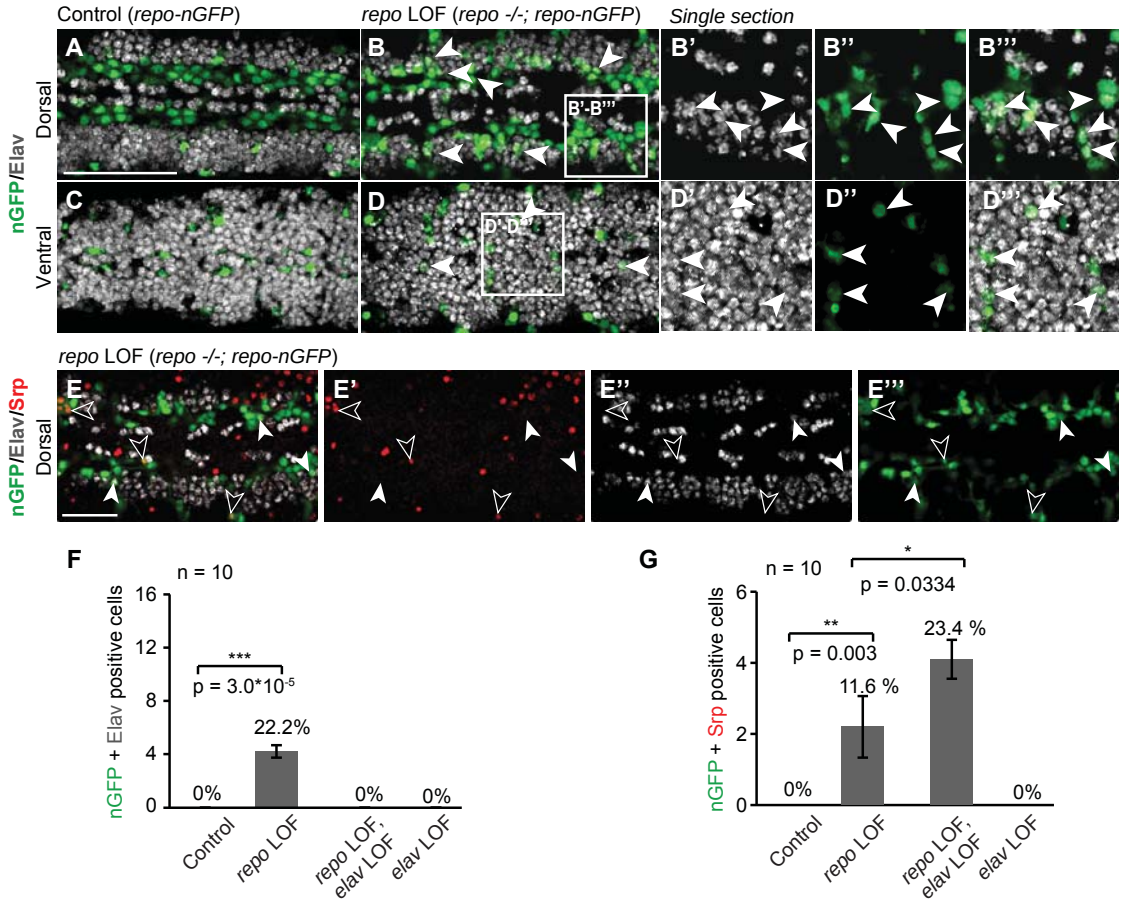
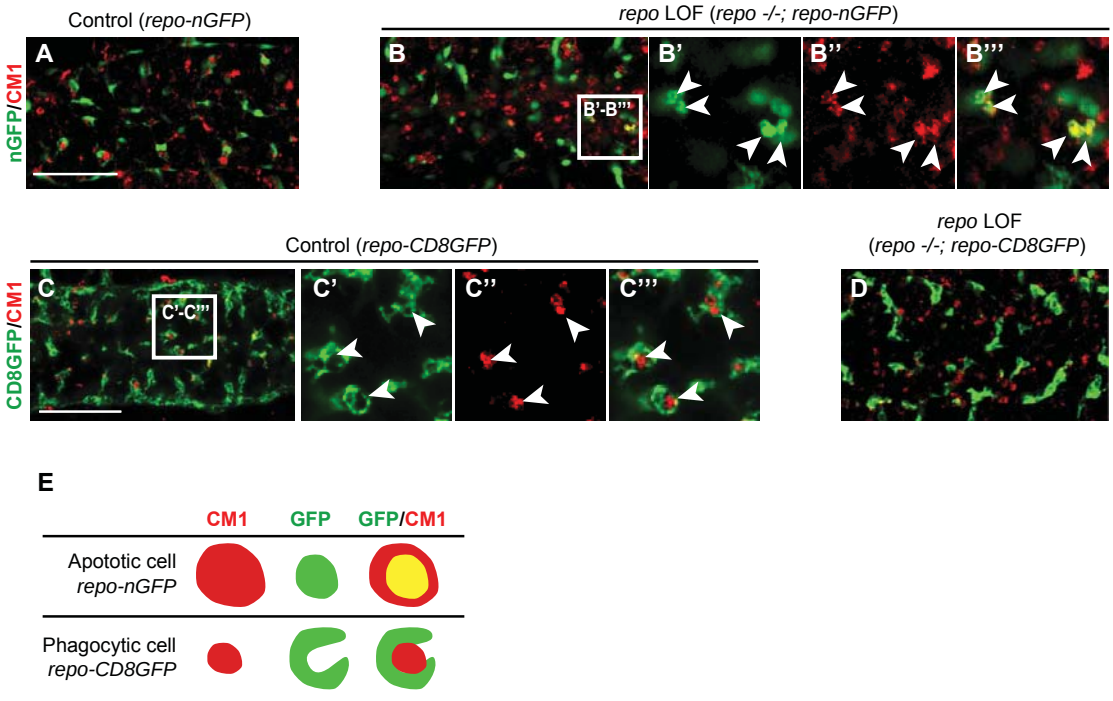

Control (repo-nGFP)
repo LOF (repo -/-; repo-nGFP)
Single section
nGFP/Elav
Dorsal
Ventral
A
B
B'-B'''
B'
B''
B'''
C
D
D'-D'''
D'
D''
D'''
repo LOF (repo -/-; repo-nGFP)
nGFP/Elav/Srp
Dorsal
E
E'
E''
E'''
F
n = 10
nGFP + Elav positive cells
***
p = 3.0*10-5
22.2%
0%
0%
0%
Control
repo LOF
repo LOF; elav LOF
elav LOF
G
n = 10
nGFP + Srp positive cells
*
p = 0.0334
**
p = 0.003
23.4 %
11.6 %
0%
0%
Control
repo LOF
repo LOF; elav LOF
elav LOF

Figure 8


Control (repo-nGFP)
repo LOF (repo -/-; repo-nGFP)
nGFP/CM1
A
B
B'-B'''
B'
B''
B'''
Control (repo-CD8GFP)
repo LOF
(repo -/-; repo-CD8GFP)
CD8GFP/CM1
C
C'-C''
C'
C''
C'''
D

E

CM1
GFP
GFP/CM1
Apototic cell
repo-nGFP
Phagocytic cell
repo-CD8GFP